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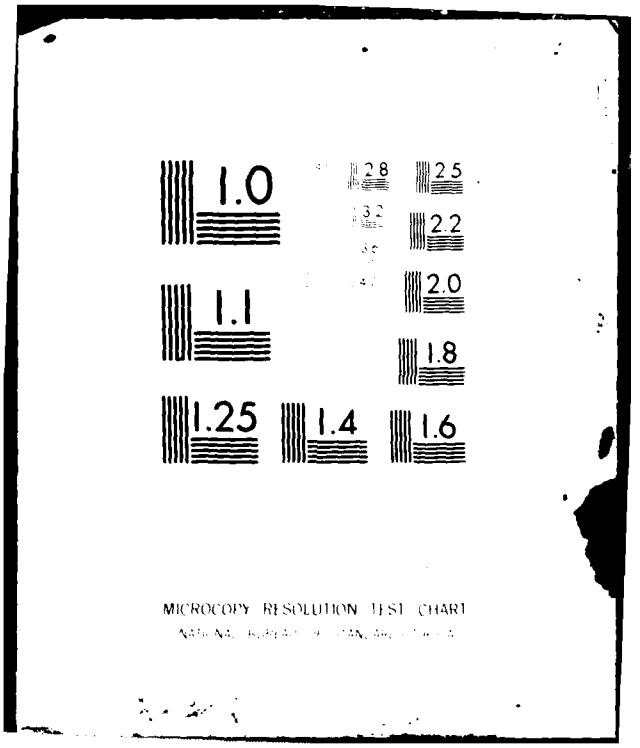
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Contract N00014-77-C-0262

Final Report

Control of the Inflammatory Response

by

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April 23, 1982

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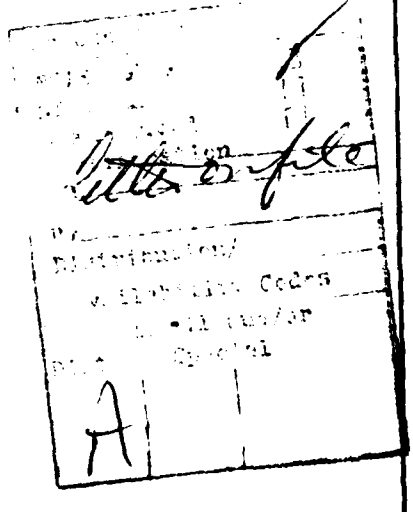
This inflammatory response is mediated and controlled by a variety of regulatory factors released by activated lymphocytes. We have found that extracts of lymphoid tissue contain factors with biological activity similar or identical to factors released from activated lymphocytes. We have identified, isolated, and characterized several mediators of inflammation from calf thymus including a T-cell specific inhibitor, a chemotactic factor fot T-lymphocytes, Macrophage Migration Inhibition Factor, and a lymphnode permeability factor. In addition, during the course of our work on the T-cell specific inhibitor, two additional inhibitors have been identified. First, a bacterial product from Pseudomonas aeruginosa and secondly, an inhibitor synthesized chemically from the polyamine spermine. The isolation and characterization of lymphocyte regulatory factors will greatly aid in understanding the mechanism of the inflammatory response. In addition, the inhibitory factors, whether from the thymus or the bacterial or synthetic product, may prove to be important in limiting the inflammatory response in situations where this would be clinically useful, such as transplantation.



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Final Report for Contract N00014-77-C-0262

Control of the Inflammatory Response

A. Summary.

Inflammation, or the response of tissue to an injury, involves the reaction of a number of cells, primarily various types of lymphocytes. These lymphocytes interact with each other and other cell types by a complex array of chemical messengers. Our research interest has been in the transmission of negative regulatory signals during inflammatory response. There are two common sources for factors which can influence the action of lymphocytes, the culture fluid of activated lymphocytes and lymphoid tissue. We have chosen to concentrate on the isolation of regulatory factors from lymphoid tissue as the amounts of material are much greater, although, for certain factors, the isolation schemes may be somewhat more difficult. In most cases, the biological activity of regulatory factors isolated from activated lymphocyte cultures and lymphoid tissue are identical (1). During the course of this contract, we have identified and characterized a factor from bovine thymus which is a specific inhibitor of T-lymphocyte function. In addition, several observations made during work with the thymus inhibitor have resulted in the serendipitous discovery of two unrelated lymphocyte inhibitory factors, one synthesized from the polyamine spermine and another produced from Pseudomonas aeruginosa. While it is impossible to eliminate the large number of external stimuli capable of eliciting an inflammatory response, it will be possible to limit the extent of the response by application of factors which specifically inhibit various types of cells participating in the response. In addition to the inhibitory factor from thymus several other important modulators of the inflammatory response, Migration Inhibitory Factor, a protein chemotatic for T-lymphocytes, lymphotactin, and lymph node permeability factor (LNPF), have been isolated and characterized.

a. Thymus-derived inhibitors.

One of the major events during the inflammatory response is the transformation of the small circulating lymphocyte into a large lymphoblast. These lymphoblasts then go on to release regulatory factors for other cells or to become effector cells. We have described previously (2-4) the isolation and preliminary characterization of a factor from thymus which inhibits lymphocyte transformation.

The thymic factor inhibits T-lymphocyte stimulation induced by alloantigens or mitogens and the spontaneous proliferation of thymocytes, but not the stimulation of B-lymphocytes by the polyclonal mitogen LPS. The stimulation of B-cells by Pokeweed Mitogen is inhibited, presumably due to the role Helper T-cells play in this response. Several cultured cell lines were resistant to the activity of the factor including both normal fibroblasts and neoplastic cells of various types. Recently, we have preliminary results which indicate that the myelopoietic leukemic cell line, K562, is relatively resistant to the thymic factor, while the lymphoblastic line, L1210, is susceptible. In summary, the thymic inhibitor is specific in its inhibitory action for cells whose maturation is thymic dependent, T-lymphocytes and thymocytes, as well as certain neoplastic cells of lymphoid origin.

The inhibitor is of low molecular weight and hydrophobic. These properties make the purification extremely difficult by conventional techniques but the rapid development of High Performance Liquid Chromatography (HPLC) technology will greatly aid in the final purification. Several other mitotic inhibitors have been characterized recently as also being low molecular weight and hydrophobic, most notably, those for granulocytes (5,6) and the JB-1 ascites cells (7).

b) Pseudomonas lymphocyte inhibitor.

We originally described a Pseudomonas bacterium contaminating lymphoid tissue extracts which contained a factor which would inhibit the transformation of human lymphocytes in the Mixed Lymphocyte Culture, the release of lymphokines in vitro, and, in preliminary experiments, the rejection of histoincompatible skin grafts in mice (8,9). A similar bacterium, identified as Pseudomonas aeruginosa, was isolated from a patient's kidney graft which also produced an immune inhibitor (9). Our biochemical evidence shows that the inhibitor was a 70-100,000 dalton protein with an isoelectric point of 6.8-7.2. This neutral isoelectric point clearly distinguishes the inhibitor from the much more acidic Pseudomonas α-toxin.

The inhibitory activity is ustable and quickly lost from even relatively crude fractions. We have altered our procedure to one involving ethanol fractionation of the bacterial extract, ion exchange chromatography on DEAE-Sephacel and gel filtration on ultragel columns. This new procedure has yielded an active fraction with a 50% inhibitory dose against MLC of 10-20 ng protein/ml. For a protein of 70-100,000 molecular weight, this is in the order of 10^{-10} M. This preparation is several times more active than our previous samples, but the activity is still unstable to procedures such as freeze-drying. The active fraction also inhibits L1210 leukemia cells at a dose of 30-40 ng/ml. This inhibitor, when injected at a dose of 5 μg/day for five days shows no toxic effect in mice. We are currently accumulating sufficient active material for detailed transplantation studies in mice.

c) Polyamine-derived inhibitors.

We have shown (3,10) that tissue and cell extracts can contain large amounts of the polyamines spermine and spermidine which can migrate on gel exclusion chromatography suggesting a much higher molecular weight than their known values. These polyamines, under certain in vitro culture conditions can act as relatively specific mitotic inhibitors (1,11). We have exploited this finding and begun the synthesis of some polyamine derivatives, produced by polymerization of spermine, which are even more potent mitotic inhibitors (12).

Our current results and conclusions, although preliminary, are as follows. The spermine-derived polycations are much more potent inhibitors of lymphoid cell proliferation than that of non-lymphoid. The method of inducing the lymphoid cell proliferation does not seem to matter and both normal and neoplastic lymphocytes are equally susceptible. The difference in sensitivity is most pronounced with the dimeric compound which is about 20 times more active against lymphoid cells than against normal fibroblasts.

In other experiments, the inhibitory activity of selected polycations of differing chemical structure and molecular size has been assayed against the MLC and L1210 leukemic cells. Several general conclusions can be made from this data.

Mainly, the activity of the spermine-derived polycations is much greater than their molecular weight (or degree of polymerization) would indicate if they are compared to the low molecular weight polybasic amino acid polylysine (-PL-12K). For example, on a molar basis, the approximately 1000 molecular weight is equally as active as the 12,000 M.W. polylysine or the 5,000-7,000 M.W. HDM, and ten times more active than the 4,000 M.W. polylysine. This may suggest that the polycationic nature is not the only requirement for inhibition and that a proper molecular size or shape can also be necessary. The further development of these spermine-derived polycations may lead to new immunosuppressive or antimitotic compounds or to drug-carriers as has been done with polylysine (13,14).

d) Migration Inhibitory Factor

We originally observed that extracts of calf thymus contained a Macrophage Migration Inhibitory Factor (MIF) which resembled that released from activated lymphocytes (15,16). This factor was purified from thymus and found to be a 35,000 dalton molecular weight glycoprotein with a pI of 6.9. The preparation was free of proteolytic activity and was not chemotactic for macrophages or other lymphocytes. The preparation did possess significant Macrophage Activation Factor activity (MAF). It would appear that a major function of the MIF/MAF would be to stimulate phagocytosis in exposed Macrophages and thus destroy any available antigens.

e) Lymphotactin

One of the most pronounced aspects of an inflammatory reaction is the infiltration of large numbers of lymphocytes into the area. This is thought to come about by the release of various chemotactic substances by the small number of lymphocytes initially reactive to the antigen or injury. We have found that extracts of thymus from calf or guinea pig which have been stimulated by PPD contain a factor chemotactic for lymphocytes (1§). This factor was active both *in vitro* and *in vivo* and may function to bring naive lymphocytes to the site of inflammation. The factor has been purified (17,18,19) and shown to be a 10,500 molecular weight sialoglycoprotein with an isoelectric point of 5.9.

f) Lymphnode permeability factor.

We have found that supernatants from transformed lymphocytes and extracts from spleen and lymph node contain a factor which increases he permeability of the microcirculation in rat skin (20,21). A similar factor can be found in extracts of bovine lung tissue (21). This LNPF is a highly anionic protein of 84,000 molecular weight and an isoelectric point of 4.2. The permeability increase is caused by the degranulation of mast cells by the LNPF and this increase is blocked by antihistamines and by the protease inhibitor pepstatin.

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sions.

xtent of the inflammatory response is controlled by numerous factors uring the reaction which transmit both negative and positive messages to ipating cells. We have found that extracts of lymphoid tissue contain rs with activity similar, or identical to, factors released by activated s. Because a large amount of lymphoid tissue can be processed at one s can be a major source of lymphocyte regulatory factors. We have l and started the characterization of a low molecular weight factor from ıs which can inhibit the transformation of small lymphocytes to lympho- This factor, which is specific for T-lymphocytes, could prove to be an potential drug to limit the extent of an inflammatory response, either r acute. Lymphoid tissue is also an important source for other factors mphotactin, MIF/MAF, and LNPF.

Accomplishments.

najor accomplishments of the research conducted under this contract are as

the identification and partial purification of a factor from calf thymus which specifically inhibits the transformation of murine or human T-lymphocytes.

the synthesis of a new class of polycations based on the polyamine spermine. These polycations may prove to be a potential new type of anti-mitotic and immunosuppressive agent.

and the purification of a bacterial product produced by Pseudomonas aerugenosa which inhibits lymphocyte transformation at the ng/ml level.

the identification, purification, and characterization of other lymphocyte function regulators from lymphoid tissue, namely, lymphotactin, MIF/MAF, and lymphnode permeability factor.